In the Specification:

[0001] This application claims the benefit under 35 U.S.C. 119(e) of provisional application U.S. Serial No. 60/413,842, filed September 24, 2002, entitled "SERA SCREEN ELISA PROTOTYPE," ~~and provisional application U.S. Serial No. 60/474,655, filed May 30, 2003, entitled "SOLUBLE HLA MOLECULES, APPARATUS AND METHODS: VLDL RECEPTOR TAGGING, ANTIBODY REMOVAL SCREENING AND IMMUNOSUPPRESSANT THERAPY",~~ the contents of which are hereby expressly incorporated herein by reference in their entirety. **This application also claims the benefit under 35 U.S.C. 119(e) of provisional application U.S. Serial No. 60/474,655, filed May 30, 2003, entitled "SOLUBLE HLA MOLECULES, APPARATUS AND METHODS: VLDL RECEPTOR TAGGING, ANTIBODY REMOVAL SCREENING AND IMMUNOSUPPRESSANT THERAPY".**

[00017] A ~~cell pharm~~ **hollow fiber bioreactor unit** is inoculated with the at least one suitable host cell containing the plasmid containing the truncated PCR product such that the ~~cell pharm~~ **hollow fiber bioreactor unit** produces soluble HLA molecules, wherein the soluble HLA molecules are folded naturally and are trafficked through the cell in such a way that they are identical in functional properties to an HLA molecule expressed from the HLA allele mRNA and thereby bind peptide ligands in an identical manner as

full-length, cell-surface-expressed HLA molecules. The individual, soluble HLA molecules are then harvested from the ~~cell pharm~~ **hollow fiber bioreactor unit** and purified substantially away from other proteins. The purification process involves affinity column purification and filtration. The purified individual soluble HLA molecules maintain the physical, functional and antigenic integrity of the native HLA molecule.

[00055] While hollow fiber bioreactor units or ~~cell pharms~~ **CELL-PHARM®s** have been described herein for utilization in the culturing methods of the present invention, it is to be understood that any large scale mammalian tissue culture system evident to a person having ordinary skill in the art may be utilized in the methods of the present invention, and therefore the present invention is not specifically limited to the use of a hollow fiber bioreactor unit or a ~~cell pharm~~ **CELL-PHARM®**.

[00070] The protocol herein discussed provides a method to couple protein to a commercially available CNBr-activated ~~Sepharose~~ **SEPHAROSE®** 4B (APB #17-0430-01). An alternative option would be running the procedure with ~~Sepharose~~ **SEPHAROSE®** 4 Fast flow (APB #17-0981-01). ~~Sepharose~~ **SEPHAROSE®** Fast Flow is more highly crosslinked than ~~Sepharose~~ **SEPHAROSE®** 4B. As a result, Fast Flow beads are more

stable and can withstand higher flow rates than the 4B beads. CNBr-activated ~~Sepharose~~ **SEPHAROSE®** 4B is better suited for batch chromatography and small columns with gravity flow. Another difference is in coupling capacities. The coupling reaction proceeds most efficiently in the pH range 8-10 where the amino groups on the ligand are predominantly in the unprotonated form. A buffer at pH 8.3 is most frequently used for coupling proteins. IgGs are often coupled at a slightly higher pH, for example in a $NaHCO_3$ buffer (0.2-0.25 M) containing 0.5 M NaCl, at pH 8.5–9.0. Carbonate/bicarbonate and borate buffer systems with the addition of NaCl may be used. The coupling buffer solution should have a high salt content (about 0.5 M NaCl) to minimize protein-protein adsorption caused by the polyelectrolyte nature of proteins. Coupling at low pH is less efficient but may be advantageous if the ligand loses biological activity when it is fixed too firmly, e.g., by multi-point attachment, or because of steric hindrance between binding sites which occurs when a large amount of high molecular weight ligand is immobilized. A buffer of approximately pH 6 is used. Tris and other buffers containing amino groups must not be used at this stage since these buffers will couple to the gel.

[00074] A titration can be performed as a first step in estimating the ratio of column matrix needed to bind a given amount of antigen. This can

be handled where an equal volume of the antibody/~~Sepharose~~ **SEPHAROSE®** 4B matrix is added to samples containing increasing concentrations of the antigen. The slurry is mixed at 4°C for 1 hr and then processed. This will yield a rough idea of the volume of column matrix needed to collect the desired amount of antigen. If the supernatants from the binding reaction are assayed for the presence of the antigen, the extent of antigen depletion also can be determined.

[00081] For biochemical analysis, monoclonal antibodies are particularly useful for identification of HLA locus products and their subtypes. W6/32 is one of the most common monoclonal antibodies (mAb) used to characterize human class I major histocompatibility complex (MHC) molecules **(ATCC® No. HB-95™, American Type Culture Collection, Manassas, VA)**. This antibody recognizes only mature complexed class I molecules. It is directed against a conformational epitope on the intact MHC molecule that includes both residue 3 of β2m and residue 121 of the heavy chain (Ladasky JJ, Shum BP, Canavez F, Seuanez HN, Parham P. Residue 3 of beta2-microglobulin affects binding of class I MHC molecules by the W6/32 antibody. Immunogenetics 1999 Apr;49(4):312-20, the contents of which is expressly incorporated herein by reference in its entirety.). Some HLA-C molecules could not be clearly identified in immunoprecipitations with

W6/32, suggesting that these HLA-C locus products may be associated only weakly with β2m, explaining some of the difficulties encountered in biochemical studies of HLA-C antigens. The polypeptides correlating with the C-locus products are recognized far better by HC-10 than by W6/32 which seems to confirm that at least some of the C products may be associated with β2m more weakly than HLA-A and -B.

[00085] The present invention is directed to a unique method for producing, isolating, and purifying class I molecules in substantial quantities. As an example of the method of the present invention, the following graphs show that the test allele B*0702BSP produced in static culture can be purified to homogeneity and eluted as intact molecule. FIG. 5 demonstrates that a W6/32-coupled affinity column can be saturated with crude harvest containing sHLA. Individual values were determined through a standardized sandwich ELISA procedure using W6/32 as capturing antibody and anti-β2m as detecting antibody. This ELISA procedure allows only the detection of intact sHLA molecules. After successful loading, the column is washed with PBS. FIG. 6 shows the washing step. The removal of total protein and active sHLA measured through OD280 and ELISA, respectively, can be followed. It shows that after 500 ml of wash volume, impurities are successfully removed from the column. This was also confirmed through SDS-PAGE

analysis of the wash fractions collected. In FIG. 7, we were able to elute sHLA molecules with 0.1 M glycine (pH 11.0) and neutralize in 1 M potassium phosphate (pH 7.0) that resulted in fractions of intact molecules as shown through the standard ELISA procedure as well as OD280 detection. Elution occurred in a single peak indicating the absence of nonspecifically bound material on the column. SDS PAGE analysis confirmed the size of the subunits and their purity. The final ~~Macrocep~~ **MACROSEP®** procedure was used to remove the neutralization buffer and replace it with PBS (0.02% Sodium azide). This buffer is highly suitable to maintain structural integrity and maintain the stability of the sHLA complex.

[00086] The same procedure is used to finally concentrate the protein to increase its stability. Higher concentrations are usually more suitable in most applications. All ~~macrosep's~~ **MACROSEP®'s** wash flow through's have minimal sHLA content and are usually discarded after the procedure. To remove possible particles or bacterial growth, filtration through a 0.2 micron filter is standard procedure. With this purification run, an overall efficiency of 75% was achieved.

[00089] The sHLA's produced and purified by the method of the present invention were analyzed by ~~Superdex~~ **SUPERDEX®** chromatography to

demonstrate sample purity (FIG. 10). The ~~Superdex~~ **SUPERDEX®**-FPLC analysis of B*1512T under native conditions showed a characteristic peak corresponding to the sHLA complex. No other major bands can be detected confirming the pure nature of our preparation. Under such native conditions, a peak of the size of 39.7 kDa is seen, which is in the area of complexed sHLA. No bands at 31 kDa, representing free HC, or at 12 kDa for β2m are visible. However, a minor band at approximately 94.5 kDa can be seen, which represent aggregated HCs. Because sHLA samples are filtered through a 10 kDa filter during the ~~Macrocep~~ **MACROSEP®** procedure, these free HC molecules remain in the solution and cannot be removed. Aggregated HC's are not considered an impurity of the sample. In addition, their contribution to the final protein amount is less than 1%. The overall purity of the complex compared to foreign proteins is more than 99.9%.

[000102] In summary, shown in FIG. 18 is a general outline of the purification and characterization procedures of soluble human HLA proteins of the present invention. The first steps involve sHLA construct design and transfection procedures followed by large scale production of sHLA molecules in cell pharms. The sHLA is then purified by affinity column purification (which includes the steps of loading, washing and elution) and buffer exchange and concentration of purified allele using ~~Macrocep~~ **MACROSEP®**

concentration filters. The pure protein is then sterile filtered, aliquoted and stored, and the concentration of the stored pure protein is determined. Finally, quality control demonstrating the extent of chemical purification is performed using techniques known to those of ordinary skill in the art, including but not limited to, SDS-PAGE, Western blot analysis, ~~Superdex™~~ **SUPERDEX®** chromatography to demonstrate sample purity, and the like.

[000106] The assay of the presently claimed invention is performed by first attaching sHLA molecules to a substrate, such as a solid support. The substrate may be any insoluble support to which the sHLA molecule can be bound, either directly or indirectly, which is readily separable from soluble material, and which is otherwise compatible with the overall methods of the present invention. The surface of such substrates may be solid or porous, and the substrates may have any shape that allows the substrate to function in accordance with the present invention. Examples of substrates that may be utilized in accordance with the present invention include, but are not limited to, microtiter plates, such as but not limited to ELISA plates; membranes, such as but not limited to, nitrocellulose membranes, PVDF membranes, nylon membranes, acetate derivatives, and combinations thereof; fiber matrix, ~~Sepharose~~ **SEPHAROSE®** matrix, sugar matrix; plastic chips; glass chips; or any type of bead, such as but not limited to,

~~Luminex~~ **LUMINEX®** beads, ~~Dynabeads~~ **DYNABEADS®**, magnetic beads, flow-cytometry beads, and combinations thereof. The substrates are typically formed of glass, plastic or any other type of polymer, such as but not limited to PVC, polyvinyl propylene, polyethylene and the like, polysaccharides, nylon, nitrocellulose, and combinations thereof. Microtiter plates are especially convenient because a large number of assays can be carried out simultaneously, using small amounts of reagents and samples. Where separations are made by magnetism, the support generally includes paramagnetic components, preferably surrounded by plastic.

In the Sequence Listing:

Please add the Sequence Listing submitted herewith to the subject application. Such Sequence Listing contains sequences that were originally disclosed in Figures 8 and 15 of the subject application, and therefore Applicants respectfully submit that the addition of the Sequence Listing does not constitute new matter. Entry thereof is respectfully requested.